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(54) Title: METHOD FOR EX VIVO PROLIFERATION AND DIFFERENTIATION OF ADULT PANCREATIC ISLET CELLS, MEDIA USEFUL THEREFOR AND USES THEREOF

(57) Abstract

A method for inducing the profileration and differentiation of neonatal and/or adult human or non-human pancreatic islets to produce a product useful, for exmple, as a therapeutic agent for treatment of diabetes has been developed. Invention method involves a series of complex cell culture media containing necessary nutrients and growth factors, a human cytokine (hepatocyte growth factor or scatter factor), a microgravity culture vessel for promoting three dimensional growth, and molecular biology assays for measuring insulin promoter activity. A method for providing a hybrid organoid comprising a combination of donor and recipient cell types is also described.

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Method for *Ex Vivo* Proliferation and Differentiation of Adult Pancreatic Islet Cells, Media Useful Therefor and Uses Thereof

Field of the Invention

The present invention relates to methods and compositions for the proliferation of adult human or non-human pancreatic islets of Langerhans. Invention methods are useful, for example, to provide a therapeutic for treatment of type 1 diabetes mellitus, wherein such cells are optionally encapsulated within alginate microcapsules before delivery to a subject. The invention employs a series of complex cell culture media containing various nutrients which are sufficient to promote long term cell growth or multiplication and to avoid senescence or loss of biological function. The invention includes methods for the proliferation of islets in the form of high fidelity three dimensional tissue-like structures employing a microgravity culture vessel. In another aspect, the invention utilizes a novel beta cell marker using molecular biology for specifically measuring the transcriptional activity of the insulin promoter in pancreatic beta cells. In yet another aspect, the invention provides a combination of donor and recipient cell types to provide an organoid with reduced immunogenic potential.

Background of the Invention

Type 1 insulin dependent diabetes mellitus is characterized by the loss of insulin producing beta cells from the pancreatic islets of Langerhans. Standard therapy has included parenteral administration of insulin (either bovine or porcine or recombinant human) by means of multiple daily injections or an indwelling catheter and pump, but this treatment can only temporarily delay the pathological complications of the disease.

Adult human pancreatic islets have been transplanted into patients to achieve independence from insulin injections. Such transplantations require the use of immunosuppressive treatment (e.g., with cyclosporine A) to prevent rejection of the transplanted cells. The use of immunosuppressive treatment has been limited, however, by toxic side effects and by increased potential for infection. Moreover, inadequate supplies of human islets and the complications of graft rejection have necessitated the search for an improved source of islet cells.

Fetal pancreatic islets contain many undifferentiated beta cells which can mature after transplantation and which are less subject to rejection by the recipient, but they cannot be obtained in large enough quantities to serve as a practical therapeutic approach. Transplantation of individual cells or cellular communities (including human or porcine pancreatic islets, hepatocytes, keratinocytes, chondrocytes, acinar cells, or chromaffin cells) will require a virtually inexhaustible supply of functional living cells for use in human therapy.

While it has been reported that proliferation may occur from fetal tissue, the ability to proliferate adult, terminally differentiated islet cells, has eluded investigators. Each mature islet of Langherhans is a cellular community comprising four distinct cell types arranged in the typical topographical distribution. At the central core are the beta cells, which secrete insulin in response to elevated glucose. At the outer peripheral rim are alpha cells (which secrete glucagon), PP cells (which secrete pancreatic polypeptide), and Delta cells (which secrete somatostain).

The ability to demonstrate ong ing physiol gical release of insulin following multifold proliferation of fully differentiated adult islet cells has eluded

investigators in the past. Attempts by previous investigators have failed due to overgrowth f the beta cells by fibroblasts, resulting in negative selection of endocrine secreting cells, and thus cessation of insulin secretion from the proliferated cells.

Accordingly, there is a clear need in the art for methods to promote the proliferation of fully differentiated adult islet cells to produce increased quantities of cells which are capable of ongoing physiological release of insulin.

Proliferation of mammalian cells presents special problems. Thus, in contrast to mammalian cells, free living microorganisms (e.g., bacteria or fungi) possess tough cell walls for resistance to most environmental stresses. In addition, such organisms synthesize nearly all of the biomolecules essential to life. However, cells from higher organisms (e.g., mammals or humans) are structurally delicate and require constant supplements of specific nutrients to maintain viability. Large scale processes are better developed and less difficult for culturing bacteria than for culturing mammalian cells. Thus, while bacterial cells can be grown under vigorous agitation in large volumes of simple liquid medium, mammalian cells, in contrast, are more difficult to grow: they are easily damaged by the shear stresses of turbulent fluid flow, they require complex nutrient media to support cell growth, and they often grow better in the presence of an appropriate substrate surface which promotes cell attachment.

As an alternative to proliferation of mature adult cells, immortal carcinoma cell lines have been developed and pr pagated for many years. Prior art techniques, however, have not permitted the growth of non-neoplastic, non-transformed cells (including adult

pancreatic islets) in large scal three dimensi nal cultures (which pr mote the important cell-to-cell contacts found in natural tissue). While small scale culture systems (e.g., plastic plates with microwells) are adequate for laboratory experiments, they do not provide enough surface area for commercial production. Certain carcinomas have been successfully grown in the presence of a pre-established stromal support matrix, which is composed of non-living material and inoculated with stromal cells. Free floating tumor cell spheroid aggregates, both with and without attachment substrates such as microcarriers, have provided material for experimental analysis of embryological development and chemotherapeutic cytotoxicity. Collagen coated cellulose sponges have allowed carcinoma cells to adhere, migrate, and proliferate on a solid substrate in the presence of fibrin, although degenerative changes were detected after ten days of culture. Microcarrier beads provide increased surface area for cellular attachment, allowing them to assemble into tissue-like three dimensional structures which mimic the natural relationships. Agitation in a conventional impeller driven bioreactor vessel suspends the cells in the medium, delivers fresh nutrients, and removes metabolic waste products, but it also subjects them to high levels of shear stresses which can damage cells and inhibit cellular tissue assembly.

Accordingly, what is needed in the art are methods to promote the proliferation of fully differentiated adult islet cells to produce increased quantities of cells which are capable of ongoing physiological release of insulin. Especially desirable would be methods for the production of such cells under conditions which promote assembly into tissue-like three dimensional structures which mimic the natural relationships of cells in native tissues.

Brief Description of the Invention

In one aspect of the present invention, it has been discovered that a specifically defined media, as well as a specifically defined environment in which the cells are cultured, allow proliferation of adult differentiated islet cells, with ongoing insulin secretion in the cells thus proliferated.

In accordance with another aspect of the present invention, there has been developed a method of proliferating human and non-human islet cells to produce huge quantities of synthetic islet cells which demonstrate continued physiological activity. The invention method is made possible by the identification of specific defined cell culture media, growth factors, differentiation factors and environmental factors which enhance the selection of beta cells. With this accomplishment, it is now possible to provide a virtually unlimited supply of insulin-secreting cells for the treatment of insulin dependent diabetic patients.

In accordance with yet another embodiment of the present invention, it has been discovered that by surrounding the cells in a physical environment which mimics that of the fetus in the pregnant uterus (i.e., by suspending the cells in a cell culture device which allows low shear as well as maximum co-localization of the cells), maximum pseudo islet (i.e., islet-like) or aggregate formation is induced, resulting in the formation of organoids with excellent secretory function and differentiation.

Having developed means to produce proliferated single beta cells, or endocrine cells, from islets, th re have further been developed means whereby these single islet cells can be reaggregated into a three dimensional

morphology resembling that of natural intact islets containing alpha, beta, delta, and PP cells. These pseudo islets are reaggregated under conditions which appear to further enhance their state of differentiation. The conditions under which this is accomplished is one of low shear using a device which provides for microgravity conditions.

The invention also describes a method of co-culturing proliferated islets from a donor pancreas, together with cell types optionally obtained from the recipient. These cell types include fibroblast, endothelial neural cells, and the like, which together may reduce the immunogenicity of the hybrid co-cultured organoid, as well as enhance the functional activity of the resulting pseudo islet. In addition, it has also been discovered that co-culturing freshly isolated, non-proliferated islets with islet cells which have undergone proliferation, and encapsulating these co-cultured organoids, results in cells having a longer viability, stability and insulin secretory activity than do either of the components of the co-cultured organoid alone. Furthermore, there is also the potential of encapsulating, together with these proliferated islets, other cell types (including acinar cells, hepatocytes, and the like) which may provide enhanced activity of these co-cultured hybrid organoids.

Further in accordance with the present invention, the somatostatin transcription factor, STF-1, has been used as an identifying marker to detect specific functional activity of the insulin promoter concurrent with insulin expression in the beta cells of pancreatic islets. This STF-1 factor is used as a probe, not only to optimize the c ll culture media in terms of developing a media which provides the highest STF-1 activity, but also provides a probe for identifying STF-1 cells, and thus insulin

secreting cells. Immunohistochemical and molecular biological techniques involving antibodies to STF-1 make it possible to monitor and analyze insulin expression at the level of transcription of DNA into mRNA within the cell nucleus.

Brief Description of the Figures

Figure 1 presents a flow chart outline of the invention method whereby adult human islet cells are produced by initiation, expansion and termination of adult human islet cell proliferation and pseudo islet formation *in vitro*.

Figure 2 presents a growth curve for proliferating human adult islet cells.

Figure 3 presents a diagram of the population doubling time of human adult islet cells generated by cell counting. About 20 doublings are required to produce 1 million cells.

Figure 4 presents a comparative bargraph representing the glucose + theophylline stimulated insulin secretion from 12,000-fold expanded islet cells after 24 h aggregation and encapsulation.

Figure 5A represents the perifusion curve of normal adult islets.

Figure 5B is a graphic presentation of the insulin secretion rate of 12,000-fold expanded adult human pseudo islets after encapsulation.

Figure 6A is a graphic presentation f insulin s cretion from co-encapsulated adult islets with 1000-fold expanded pseudo islets.

Figure 6B is a graphic presentation of insulin secr tion from encapsulated proliferated islets alone. Comparison of Figures 6A and 6B demonstrate enhanced viability and function of co-cultured islets.

Detailed Description of the Invention

In accordance with the present invention, there is provided a method for stimulating the *ex vivo* proliferation and differentiation of neonatal and/or adult pancreatic islet beta cells, said method comprising:

(a) contacting a primary culture of neonatal and/or adult pancreatic cells under conditions suitable to induce beta cell proliferation; and

(b) contacting the differentiated cells produced in step (a) under conditions suitable to induce prolonged proliferation of said cells.

As employed herein, "primary culture" refers to a mixed cell population of neonatal and/or adult pancreatic cells which permits interaction of epithelial and mesenchymal cells within islet-like cell clusters.

As employed herein, "*ex vivo*" refers to cells which have been taken from a body, temporarily cultured *in vivo*, and then returned to a body.

As employed herein, "proliferation" refers to an increase in cell number.

As employed herein, "differentiation" refers to increased numbers of islet-like cell clusters containing an increased proportion of beta epithelial cells which produce increased amounts of insulin per cell.

Pancreatic tissue source suitable for use in the practice of the pr sent invention includ adult human

pancreases (which can be obtained from cadaver organ donors, as well as living donors), neonatal human pancreases, neonatal and/or adult porcine pancreases, and the like. Non-human adult pancreata are obtainable from porcine or bovine sources. Pancreata are typically shipped on ice in standard medium (e.g., RPMI 1640, Irvine Scientific, Irvine, CA) supplemented with 10% normal human serum and antibiotics (penicillin 100 U/ml, streptomycin 0.1 mg/ml, and amphotericin B 1 mg/ml), and are processed within 6 to 12 hours of retrieval.

Pancreatic islet isolation can be carried out as known in the art, for example, by digesting human and non-human adult pancreata using collagenase (e.g., collagenase P, Boehringer, Indianapolis, IN) under aseptic conditions (see, for example, Soon-Shiong et al., in *Transpl.* 54:769-774 (1992)). Islets are purified by gradient technical separation, viability tested by acridine orange/propidium iodide uptake, and beta cell concentration estimated via a specific vital dye stain (dithazone) i.e., DTZ uptake.

Explants subjected to tissue culture in accordance with the present invention may consist of highly purified adult islets (human or porcine), or of adult islets mixed with exocrine or duct tissue, or of disaggregated single cells of highly purified adult islets.

Islet cell proliferation is initiated from highly purified whole islets, instead of monodispersed islet cells. The advantage of not starting with single cells can be explained with reference to both the physiology and anatomy of the islet microorgans (see, for example, Pipeleers et al., in *Proc. Natl. Acad. Sci. USA* 79:7322-7325 (1982), Cell Biology Section). Glucose-induced insulin release depends on functional cooperation between islet cells. Exposure to glucose caused release of 30-fold more insulin from beta cells lodged within intact islets as

from purified single beta cells. Structurally coupled beta cells and single beta cells isolated with alpha cells responded 4-fold more effectively to glucose than single beta cells. Glucose responsiveness is dependent not only the number and integrity of insulin containing beta cells, but also their interactions with their neighboring beta and non-beta cells. Insulin secretion is seen to depend on the micro anatomy and functional organization of the islets. (Pipeleers et al., supra).

The solid growth substrate may be a surface-treated polystyrene petri dish (FALCON 3003), a tissue culture flask, or the like, coated with a variety of agents (e.g., Matrigel, laminin, fibronectin, collagen, hyaluronic acid, and the like) for selective attachment. These solid growth substrates may be re-used after trypsinization. Islet cells may be co-cultured with fibroblast cells. The differentiated state is induced by extracellular matrix, by growth factors, or by contact with neighboring cells. The differentiated state is stabilized by cell-cell adhesion, cell-cell communication, cell substrate adhesion, cell substrate interaction, and the like.

Various nutrients and factors supplemented into the growth medium are critical for the long term viability and health of mammalian cells in culture. In accordance with the present invention, a combination of ingredients in appropriate amounts has been demonstrated to enhance the proliferation of adult pancreatic islets, thereby providing a virtually unlimited supply of therapeutic material, exceeding the supply available from natural sources. In order to induce differentiated adult insulin secreting cells to proliferate and maintain their physiological status, it has been discovered that a molecular environment simulating that of the pregnant state must be provided to the cells in culture. This molecular environment includes factors which 1) induce specific growth of beta cells

(scatter factor), 2) induce cellular growth and mitosis, 3) promote differentiation (nicotinamide), and 4) inhibit fibroblast overgrowth. All of these factors, in combination, are critical to achieving proliferated beta cells which are capable of insulin secretion following multiple fold mitosis.

Suitable culture medium is prepared using a standard commercially available cell culture medium (e.g., RPMI DMEM, Ham's F12) as a base, at a pH of about 7.4, containing an effective cell growth promoting concentration of water, calcium ions, sodium ions, glucose, insulin, transferrin, all essential amino acids, water soluble vitamins, coenzymes, and glucose. The culture medium should contain a source of an aqueous mixture of lipoprotein, cholesterol, phospholipids, and fatty acids with low endotoxin. A broad spectrum antibiotic (e.g., gentamicin) can also optionally be included in the culture medium to prevent contamination by bacteria, yeast, or fungi.

Hepatocyte growth factor is added to the culture medium to stimulate the proliferation of adult pancreatic beta cells from primary cultures of adult pancreatic cells, to increase insulin production in primary and secondary cultures of adult pancreatic islet cells, and to prepare large quantities of functional adult pancreatic beta containing islets for transplantation into diabetic patients.

Hepatocyte growth factor (HGF or scatter factor) is an 87 kDa two chain glycoprotein cytokine, a potent hepatocyte mitogen, and a fibroblast secretory protein which increases the motility of epithelial cells. It has been purified to h mogeneity, sequenced, and genetically cloned. It was identified immunohistochemically in pancreatic glucagon s creting A cells (but not exocrine

cells) of adult humans or rats, and also in developing pancreatic acinar cells of fetal rats.

In accordance with the present invention, nicotinamide is added to cell growth media at the appropriate concentrations and at the appropriate stages of the proliferation, thereby inducing specific beta cell differentiation. While nicotinamide has been discovered to be toxic to cells when present at too high a concentration, it has also been discovered that the presence of nicotinamide at the appropriate stages of the culture period serves not only to desirably inhibit fibroblast overgrowth, but also to desirably induce beta cell differentiation and increase insulin content and output.

In addition, in accordance with the present invention, it has been discovered that in order to induce proliferation in adult differentiated cells, it is important to provide hormones which mimic the pregnant state. These hormones include human placental lactogen, hormones of the pituitary (including corticotropin, somatotropin, oxytocin, vasopressin, and the like), as well as hormones provided from hypothalamic extracts (e.g., growth hormone releasing hormone, thyrotropin releasing hormone, corticotrophin releasing hormone, gonadotropin releasing hormone, luteinizing releasing hormone, prolactin releasing hormone, adrenocorticotropic hormone, thyrotropin stimulating hormone, follicle stimulating hormone, luteinizing hormone, and the like). The presence of these hormones in the mileau during the proliferation phase have been shown to induce proliferation of terminally differentiated cells. In the absence of these factors, a significant reduction, and even absence, of growth in these differentiated cells is observed. An important aspect of this invention is the discovery f methods which simultaneously inhibit fibroblast overgrowth, while at the same time specifically induce (through the addition of the

hormones and growth factors described above) selection and proliferation of endocrine secreted cells. Fibroblast inhibition is accomplished by significantly lowering the serum concentration, as well as by the addition of nicotaminide.

Thus, growth media contemplated for use in the practice of the present invention are established at a pH of 7.4, an osmolarity between 270 and 320 mOsmol, a temperature of 37°C, and surface tension sufficiently low to prevent formation of air bubbles. The media contain an effective cell growth promoting concentration of water, sodium ions (Na^+), potassium ions (K^+, 0.23 g/L), calcium ions (Ca^{++}, between 0.37 and 1.1 mM), magnesium ions (Mg^{++}), zinc ions (Zn^{++}), chloride ions (Cl^-), sulfate ions (SO_4^-), bicarbonate ions (HCO_3^-), glucose (1500 mg/L), all essential amino acids, cysteine, tyrosine, glutamine (between 2 and 7 mM), water soluble vitamins, nicotinamide, coenzymes, and inorganic trace elements. Glucose is preferably present at 0.8 to 1.2 mg/mL. The culture medium contains a source of an aqueous mixture of lipoprotein, cholesterol, phospholipids, and fatty acids with low endotoxin. A broad spectrum antibiotic (e.g., gentamicin) can optionally be included in the culture medium to prevent contamination by bacteria, yeast, or fungi. The media described in the present invention are of various types for use at different stages of the proliferation and differentiation process. They include:

I. VRX-E: Establishing media for endocrine cell selection (see Example 1).

II. VRX-S: Beta cell specific differentiation media containing various concentrations of nicotinamide (see Example 2).

III. VRX-P: Extended proliferation media for extended propagation. This medium contains a reduced dose of nicotinamide, and optionally no scatter factor.

IV. VRX-C: Media to bring about cessation f proliferation whereby growth factors are removed from the base medium.

V. VRX-A: Media utilized for the aggregation of pseudo islets.

Growth factors, hormones and differentiation factors contemplated for use in the above-described media include pregnancy hormones (e.g., lactogen), gastrointestinal hormones (e.g., gastrin or CCK), pituitary hormones (e.g., prolactin or growth hormone), steroid hormones, thyroid hormones (e.g. T_3 or T_4), insulin (as Na insulin monomer), epidermal growth factor (EGF), hepatocyte growth factor (HGF), fetal bovine serum (FBS) 4%, attachment factors, spreading factors, binding proteins, and the like.

Gas phase employed for the above-described culturing is introduced as follows: The culture is perfused with a gas mixture comprising either 5% CO_2 plus 95% air plus 2.5 ng/mL selenous acid in a CO_2 incubator, or 95% O_2 plus 5% N_2. The gas temperature is maintained at 37°C and the relative humidity is maintained at 90%.

Optionally, the above-described method further comprises:

(c) contacting the proliferated cells produced in step (b) under conditions suitable to arrest the growth thereof, and thereafter, optionally

(d) culturing the cells produced in step (c) under conditions suitable to promote the formation of three dimensional tissue-like structures.

Conditions suitable to arrest the growth of said cells comprise culturing the differentiated/proliferated cells in growth cessation media, VRX-C.

Conditions suitable to promote the formation of three dimensional tissue-like structures comprise culturing cells in a microgravity device in the presence of aggregation media, VRX-A. Thus, in accordance with the present invention, it has been discovered that the microgravitational process greatly enhances the capability to form and maintain three dimensional islet clusters. It has been discovered that by using a rotational wall vessel (such as, for example, the device disclosed by Schwarz et al., in U.S. Patent No. 4,988,623, or the device disclosed by Schwarz et al., in U.S. Patent No. 5,026,650, both of which are hereby incorporated by reference herein in their entirety), the low gravity process simultaneously minimizes the fluid shear stress, provides three dimensional freedom for islet cell cluster and substrate spatial orientation, and increases localization of the various cell types of the islet (i.e. Delta, Beta and PP cells) in a similar spatial region for significant periods during the cell culture, thereby increasing the pseudo islet formation. Cells and substrate rotate about an axis nearly perpendicular to gravity. Cells of greatly different sedimentation rates orbit in particular paths and remain spatially localized for many minutes or hours. This allows individual islet cells sufficient interaction time to form multicellular structures and to associate with each other. A vessel diameter is chosen which has the appropriate volume for the intended quantity of cultured material and which will allow a sufficient seeding density of cells, tissues, and substrates. The outward particle drift due to centrifugal force is exaggerated at higher vessel radii and for rapidly sedimenting particles. Selected levels of shear stress may be introduced into the culture environment by differential rotation of the vessel components, as a means for controlling the rate and size of tissue formation and for maintaining optimal particle sizes and ass ciated sedimentation rates.

Individual pancreatic islet cells cultured under microgravity conditions lead to the formation and maintenance of three dimensional aggregates possessing similar morphology and anatomical structure to that normally found in natural tissue. Thus, islet cells (after several fold expansion) were introduced into a microgravity vessel containing culture medium, growth factors, and an attachment matrix. Simulated microgravity was created (in ordinary unit gravity) by modulating the horizontal rotation of a culture vessel completely filled with culture medium containing the matrix. These conditions cause cells to co-locate in one spatial region and encourage the maintenance of aggregates because shear stresses arising from the relative motion of the medium with respect to the walls of the vessel are minimized.

Utilizing the above-described microgravity vessel technique, it has also been discovered that it is possible to co-culture proliferated islet cells, together with cells of other types (e.g., freshly isolated islets, endothelial cells, acinar cells, neural cells and cells from other solid organs such as hepatocytes, and the like). Enhanced activity of cells co-cultured in this manner has been demonstrated. The advantages of such co-cultured cells may be in their reduced immunogenic profile, as well as in their enhanced differentiated state, resulting in an enhanced secretion of the desired hormone or peptide.

In accordance with another embodiment of the present invention, there is provided a method for treating a subject with type 1 diabetes mellitus, said method comprising:

(a) contacting a primary culture of neonatal and/or adult pancreatic cells under conditions suitable to induce beta cell proliferation;

(b) contacting the differentiated cells produced in step (a) under conditions suitabl to induce prolong d proliferation of said cells;

(c) contacting the proliferated cells produced in step (b) under conditions suitable to arrest the growth thereof;

(d) culturing the cells produced in step (c) under conditions suitable to promote the formation of three dimensional tissue-like structures containing increased numbers of insulin producing islet-like cell clusters containing beta epithelial cells;

(e) encapsulating said cells or islet-like cell clusters; and

(f) parenterally administering an effective amount of said encapsulated cells to said subject.

In accordance with yet another embodiment of the present invention, there is provided a method to proliferate or differentiate neonatal and/or adult pancreatic islet cells in clinically useful quantities, said method comprising:

(a) seeding a primary culture of neonatal and/or adult pancreatic cells into a microgravity vessel;

(b) contacting said cells in said microgravity vessel with a complete growth medium supplemented with a proliferation inducing amount of a cytokine (hepatocyte growth factor) for a time sufficient to allow differentiation, proliferation and aggregation of said cells; and thereafter

(c) harvesting the resulting islet-like cell clusters containing beta epithelial cells from said microgravity vessel; and optionally

(d) encapsulating said islet-like cell clusters.

Encapsulation is opti nal becaus of the low immunogenicity of cells prepared as described herein.

In accordance with still another embodiment of the present invention, there is provided a composition for transplanting functional neonatal and/or adult pancreatic tissue into patients, said composition comprising:

a pharmaceutically acceptable vehicle, containing primary cultures of neonatal and/or adult pancreatic islet cells which have been contacted *ex vivo* with a differentiation and proliferation inducing amount of a cytokine (hepatocyte growth factor) sufficient to induce an increase in cell number, an increase in the formation of islet-like cell clusters containing beta epithelial cells, and retain the ability to produce insulin in response to stimulus.

Exemplary pharmaceutically acceptable vehicles include alginate microcapsules, as well as sterile aqueous or non-aqueous solutions, suspensions, or emulsions. Examples of non-aqueous vehicles are propylene glycol, polyethylene glycol, vegetable oils, such as olive oil and corn oil, gelatin, and injectable organic esters such as ethyl oleate. Such dosage forms may also contain adjuvants such as preserving, wetting, emulsifying, and dispersing agents. They may be sterilized, for example, by incorporating sterilizing agents into the compositions, by irradiating the compositions, or by heating the compositions. They can also be manufactured in the form of sterile water, or some other sterile injectable medium immediately before use.

In accordance with a still further embodiment of the present invention, there is provided a composition comprising a combination of freshly isolated islet cells and proliferated neonatal and/or adult islets. Such compositions are optionally encapsulated to facilitate administration to a patient. Thus, for example, such compositions can be employed for treating a subject with type 1 diabetes mellitus. This is accomplished by

parenterally administering an ffective amount of the above-described optionally encapsulated combination of cells to said subject.

In accordance with a further embodiment of the present invention, there are provided methods to identify proliferated and/or differentiated cells as beta cells, said method comprising monitoring said cells for the expression of STF-1. Such method can also be employed to optimize culture media for the differentiation of epithelial cells for selection for insulin-secreting beta cells. In this aspect, a cell population is monitored for the presence of STF-1 as a function of variations in the culture media, and the media then modified so as to maximize expression of STF-1.

Immunohistochemistry and immunocytochemistry: Phenotyping of proliferating cells is accomplished using immunochemical techniques directed against insulin, glucagon, somatostatin, SF receptor, STF-1, vimentin, and Factor VIII.

Evaluation of differentiated, proliferated islets: The polymerase chain reaction (PCR) can be used to phenotype proliferated cells. Function *in vitro* is assessed by sterile static glucose stimulation of adherent cells and by dynamic glucose stimulation to modulate the insulin secretion rate. Cell aggregation and pseudo islet formation is performed in the microgravitation system. Function *in vivo* is assessed by transplantation of encapsulated islet cell aggregates into mice with autoimmune diabetes.

Somatostatin transcription factor (STF-1) regulates insulin expression in beta cells of pancreatic islets. It stimulates the insulin gene by recognizing two islet specifying elements on the insulin promot r.

Specification of th four islet cell types (secreting ither insulin, glucagon, somatostatin, or pancreatic polypeptide) during development may be partially determined by the expression of STF-1 relative to other islet cell factors, since the development of endocrine cell types in the pancreas is believed to involve the progressive restriction of pluripotent stem cells.

STF-1 is a member of the homeobox class of transcription factors and is required for pancreatic organogenesis (Jonsson et al., *Nature* 371:606-609 (1994)). It is expressed during early development by the epithelial cells of the gut and most of the cells that will eventually form the pancreas. However, in the adult, STF-1 expression is lost in pancreatic ductal, exocrine, and alpha cells, and is restricted to the duodenal epithelium, beta cells, and a subset of delta cells (Guz et al., *Development* 121:11-18 (1995)). In adult beta and delta cells, STF-1 is required for the hallmark phenotype of these cells, the expression of insulin in beta cells (Peers et al., *Mol Endocrinol* 8:1798-1806 (1994)) and somatostatin in delta cells (Leonard et al., *Mol Endocrinol* 7:1275-1283 (1993)). STF-1 binds to the CT2 box in the human insulin promoter (Petersen et al., *Proc Nat Acad Sci USA* 91:10465-10469 (1994)) resulting in increased transcription of the insulin gene. In accordance with the present invention, STF-1 is utilized as both a marker for mature islet cells and as a requirement for insulin expression. Further in accordance with the present invention, culture conditions are optimized based upon STF-1 expression and activity in conjunction with measurements of glucose responsive insulin release.

In accordance with a still further aspect of the present invention, there is provided a method for the preparation of cell clusters from proliferated, differentiated, growth arrested cells, said method

comprising subjecting said cells to aggregation conditions in a microgravity vessel, such as, for example, the device disclosed by Schwarz et al., in U.S. Patent No. 4,988,623, or the device disclosed by Schwarz et al., in U.S. Patent No. 5,026,650, both of which are hereby incorporated by reference herein in their entirety.

The invention will now be described in greater detail with reference to the following non-limiting examples.

Example 1

This example describes the process of establishing a primary culture from highly purified adult islets and a method to induce endocrine cell selection through the use of specific defined media (VRX-E: "primary establishing media") in a cell culture environment which allows for adherence of endocrine cells and negative selection for fibroblasts.

In accordance with the present invention, it has been discovered that in order to accomplish endocrine cell selection of terminally differentiated, adult cells, as well as to induce proliferation of such cells, it is necessary to provide an environment which simulates that of the pregnant state, as well as to provide specific growth factors for beta cell selection. In addition, an environment is provided whereby such endocrine cells could attach either to a specifically coated tissue culture flask with materials such as matrogel, hyaluronic acid, laminin, and collagen. The media, which is described below, reflects the discovery that growth factors mimicking that of the pregnant state, such as human placental lactogen, growth factors from xtracts of the pituitary and hypothalamus, and factors specific for beta cell of selection (hepatocyte growth factor), are critical.

Establishing media (VRX-E) is the base m dia set forth in Table 1, containing components 1-58 thereof. Derivatives or variants of the base media are then prepared by adjusting (either by addition or subtraction or a combination thereof) the base media, for example, by adding one or more of the optional components 59-61 and/or by deletion of such additives as (13), (56), (57) and/or (58).

Table 1

MANDATORY COMPONENTS:		
1.	Glucose	1.0000 g/L
2.	Hypoxanthine	0.0047 g/L
3.	Folic acid	0.0010 g/L
4.	Thymidine	0.0007 g/L
5.	Transferrin	5.0 μg/ml
6.	Insulin	10.0 μg/ml
7.	Hydrocortisone	3.5 ng/ml
8.	Selenous acid	2.5 ng/ml
9.	Calcium chloride	0.03885 g/L
10.	Magnesium sulfate$\cdot 7H_2O$	0.060 g/L
11.	Magnesium chloride	0.060 g/L
12.	Human placental lactogen	0.001-0.020 g/L
13.	Hypothalamus extract	0.03-0.10 g/L (protein)
14.	L-alanine	0.018 g/L
15.	L-arginine HCl	0.422 g/L
16.	L-asparagine anhydr.	0.030 g/L
17.	L-aspartic acid	0.026 g/L
18.	L-cystine HCl$\cdot H_2O$	0.070 g/L
19.	L-glutamic acid	0.030 g/L
20.	L-glutamine	0.292 g/L
21.	L-glycine	0.016 g/L
22.	L-histidine HCl$\cdot H_2O$	0.042 g/L
23.	L-isoleucine	0.008 g/L
24.	L-leucine	0.026 g/L
25.	L-lysine HCl	0.073 g/L
26.	L-methionine	0.009 g/L

27.	L-phenylalanine	0.010 g/L
28.	L-proline	0.070 g/L
29.	L-serine	0.021 g/L
30.	L-threonine	0.024 g/L
31.	L-tryptophan	0.004 g/L
32.	L-tyrosine di-Na salt	0.016 g/L
33.	L-valine	0.023 g/L
34.	L-ascorbic acid	0.045 g/L
35.	Biotin	0.07 mg/L
36.	D-Calcium pantothenate	0.5 mg/L
37.	Choline Chloride	0.014 g/L
38.	Myo-inositol	0.036 g/L
39.	Niacinamide	0.04 mg/L
40.	Pyridoxine HCl	0.06 mg/L
41.	Putrescine·2HCl	0.3 mg/L
42.	Riboflavin	0.04 mg/L
43.	Thiamine HCl	0.29 mg/L
44.	Vitamin B-12	1.4 mg/L
45.	Na-pyruvate	0.220 g/L
46.	Linoleic acid	0.09 mg/L
47.	Lipoic acid	0.2 mg/L
48.	Phenol red	1.2 mg/L
49.	Sodium chloride	7.53 g/L
50.	Potassium chloride	0.23 g/L
51.	Disodium phosphate (anhydr)	0.135 g/L
52.	Potassium phosphate (monobasic)	0.068 g/L
53.	Cupric sulfate·$6H_2O$	0.002 mg/L
54.	Ferrous sulfate·$7H_2O$	0.8 mg/L
55.	Zinc sulfate·$7H_2O$	0.4 mg/L
56.	Serum (fetal, bovine or human)	10 - 50 ml/L
57.	Pituitary extract	0.01-0.05 g/L (protein)
58.	Hepatocyte growth factor/scatter factor	5-15 ng/ml

OPTIONAL COMPONENTS:

59.	Nicotinamide	2.5-10 mM
60.	Human serum albumin	1 g/L
61.	Supplemental Calcium chloride	0.078 g/L

Islets were isolated from a pancreas retrieved from a 22 year old cadaver male donor. Following collagenase digestion, the islets were purified by density gradient centrifugation, and cultured overnight in standard cell culture media containing RPMI 1640 (Biowhittakes, Inc.) supplemented with 10% fetal bovine serum. Following 24 hours culture, the islets were collected, washed and then placed in cell culture vessels which allowed attachment, optionally including tissue culture flasks coated with either matrogel, laminan fibrinogen or standard petri dishes. The cells were fed every three to four days with 5-15 mM of VRX-E establishing growth media for a period of 14 days. Endocrine cells rapidly attached to the surface of the tissue culture vessel within 24-48 hours. By the end of 14 days a confluent cell monolayer is noted. Using both histochemical and molecular biology probes, it was confirmed that this population of cells was, indeed, endocrine cells and hormone producing. The probes used to confirm the presence of endocrine cells were mRNA for STF-1, mRNA for insulin, and immunohistochemical chemistry for anti-insulin, anti-somatostatin and anti-glucagon. In addition, as described below, functional assays using static glucose stimulation, and perifusion of the cells, using glucose as a stimulus, demonstrated excellent physiological release of insulin, confirming the proliferation of insulin secreted beta cells.

Using this method of endocrine cell selection from freshly isolated islets, islet cell proliferation was confirmed in 57 different HLA typed, HIV negative, pancreatic donors between the ages of 15 to 65 yrs. Normal

Chromosomal analysis of the islet cells proliferated at various stages of doublings demonstrated that, indeed, these cells were primary cell cultures capable of propagation without transformation.

Following establishment of endocrine cell selection, the endocrine cells thus selected were frozen by cryopreservation, and stored in liquid nitrogen as a master cell bank, and are used for further expansion in subcultures.

Example 2
Beta Cell Specific Differentiation Media: VRX-S.

Following endocrine cell selection from the freshly isolated adult islet population, as described in Example 1 above, ongoing differentiation of these cells, to retain the insulin secretory capacity, must be retained. A method to specifically induce differentiation and ongoing proliferation of the endocrine cells thus selected has been developed utilizing VRX-S media. Specifically it is the goal of this process to maintain the differentiation pathway in the direction of insulin secreting cells. It is presently believed that within the cell population selected in Example 1 above, there exist multipotential progenitor cells which can be directed to differentiate into specific insulin secreting beta cells. In accordance with the present invention, it has been discovered that a high dose nicotinamide during the early phase of proliferation is critical to induce beta cell differentiation. While it has previously been described that a high dose of nicotinamide is a potent inducer of endocrine differentiation in the *fetal* human pancreas, there has been no clear evidence of the effect of nicotinamide on *adult* islet cells.

In accordance with the present invention, it has been discovered that the timing and the concentration of

nicotinamide application is critical to successful proliferation and differentiation of islets. Thus, it has been discovered that high doses of nicotinamide are needed during the early phases of the culture period to induce differentiation, but if such high doses are maintained during the extended proliferation phase (described below in Example 3), nicotinamide is observed to be toxic to the cells and is, therefore, highly undesirable at this specific stage of cell growth. Thus, not only is the nicotinamide dose important, but the timing of nicotinamide application is also seen to be very important during the growth phase of induced differentiation. This observation has not been previously reported in the art, and gives rise to the beta cell specific differentiation media defined below, utilizing different concentrations of nicotinamide.

Thus, VRX-S (specific beta cell proliferation medium) is formulated by the addition of the following amounts (g/L) of nicotinamide to 1000 ml of VRX-E (to produce the concentrations shown in parenthesis below):

VRX-S1	1.22 (10.0 mM)
VRX-S2	0.61 (5.0 mM)
VRX-S3	0.305 (2.5 mM)

Glucose stimulated insulin secretion in adult human islet cells cultured in consecutive subcultures was examined, with and without nicotinamide. After 14 days of primary culture utilizing the VXR-E establishing media described above, the cells were reseeded in VRX-S media, in the presence and absence of nicotinamide (10 mM), and subcultured for five weeks with weekly passaging.

Triplicate cultures (0.25 M cells/56 cm^2 petri dish) were tested. On day four of each passage, a sterile static glucose stimulation (SGS) test was perf rmed.

The sterile static glucose stimulation test was carried out as follows: First the cells were washed with glucose free Krebs Ringer bicarbonate (KRB) buffer (containing 100 mg/ml human serum albumin), then incubated with basal 60 mM glucose (KRB 60) buffer at 37°C in a CO_2 incubator. After 60 minutes the supernatants were saved and replaced with 350 mg/ml glucose (KRB 350) buffer for the next 60 minutes. Incubation was completed with a final 60 minutes KRB 60 exposure. Supernatants were tested for insulin content by radio immunoassay (RIA). Following the test, cells were fed with fresh medium and cultured for three more days before passaging. On day seven, cells were trypsinized, counted and reseeded with the same density (0.25 M/plate). Insulin concentration is calculated in μIU/1.0 million cells/60 min.

RIA analysis demonstrated that the cells responded to glucose by insulin secretion as shown in Table 2.

Table 2

Insulin secretion (μIU/1.0 million cells/60 min.)

	with Nicotinamide			without Nicotinamide		
expansion x	KRB-60/1	KRB-350	KRB-60/2	KRB-60/1	KRB-350	KRB-60/2
19	28	297	37.8	13	128	15.2
256	6.6	42	5.3	5.5	38	5.2
1,448	2.8	19	2.6	2.3	24	2.7
14,263	2.2	8.9	2.5	2.1	12.8	1.8
131,072	2.1	3.2	2.7	3.1	9.9	3.7